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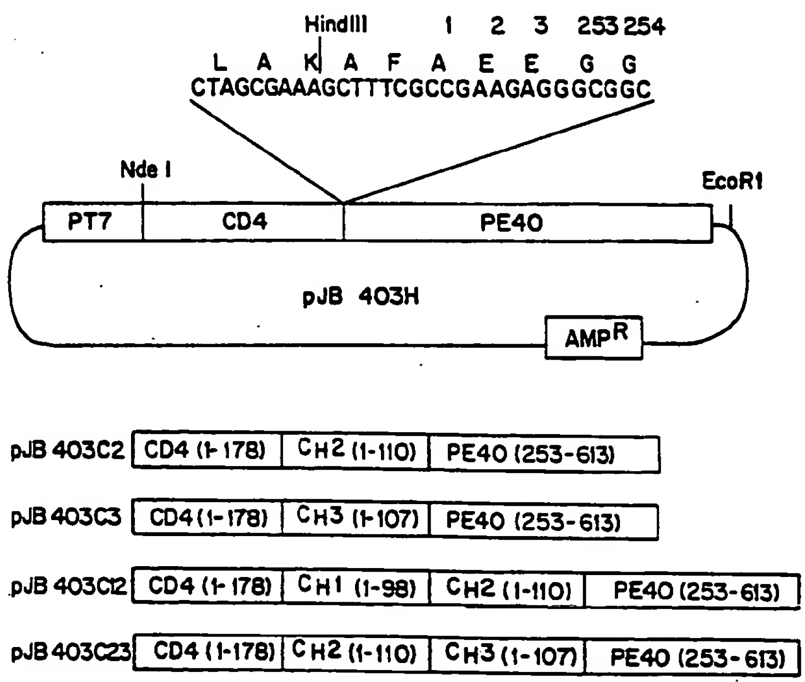

(57) Abstract

The present invention relates to the production and use of recombinant toxins modified to increase their half-life and thereby increasing their potency during therapy. More particularly, this invention relates to the use of regions of the Fc portion of an immunoglobulin molecule to confer an increased half-life on single chain chimeric toxins that include a ligand binding domain such as from CD4 receptor and a cytotoxic domain such as from *Pseudomonas exotoxin* A.

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RECOMBINANT TOXIN WITH INCREASED HALF-LIFE

FIELD OF THE INVENTION

The present invention relates to the production and use of recombinant toxins modified to increase their half-life and potency during therapy. More particularly, this invention relates to the use of specific regions of the Fc portion of an immunoglobulin molecule to confer an increased half-life on single chain chimeric toxins.

BACKGROUND OF THE INVENTION

Toxins attached to growth factors, antibodies and other cell targeting molecules can be used to kill cells bearing specific receptors or antigens (Pastan et al., *Cell* 47:641 (1986) and Vitetta et al., *Science* 238:1098 (1987)). Initially, immunotoxins were made by coupling monoclonal antibodies to toxins by chemical methods to form protein dimers.

Using recombinant DNA techniques, it is possible to make, in *E. coli*, single chain immunotoxin containing the antigen combining region (Fv) of an immunoglobulin. The antigen combining region permits efficient targeting of the immunotoxin to cells expressing the antigen recognized by the Fv immunoglobulin region. Fusing the Fv portion of an antibody to a particular cytotoxic region of a toxin can result in a chimeric protein with bi-functional domains. When the Fv region of an antibody was combined with a fragment of *Pseudomonas* exotoxin A (PE), the resulting chimeric single chain molecule was more active than the immunoconjugate of *Pseudomonas* exotoxin-coupled to a monoclonal antibody (Chaudhary et al., *Nature* 339:394 (1989) and Batra et al., *Mol. Cell. Biol.* 11:2200 (1991)).

Other recombinant toxins have been made in *E. coli* in which growth factors like TGFα, IL2, IL4, IL6, IGF1, or CD4 are fused to recombinant forms of the *Pseudomonas* toxin.

These chimeric toxins possess specific *in vitro* and *in vivo* cytotoxic activities (Pastan et al., *Ann. Rev. Biochem.* 61:331-354 (1992)).

One single chain chimeric toxin of particular interest is the CD4-PE40 immunotoxin. CD4-PE40 is a recombinant toxin consisting of a portion of the CD4 molecule (amino acids 1-178) attached to PE40. PE40 is a recombinant fragment of the *Pseudomonas* exotoxin that lacks the cell binding domain of PE (Chaudhary et al., *Nature* 335:369 (1988)). PE-40 contains the domains of PE that permit cell membrane translocation and inhibition of protein translation through the ADP ribosylation of elongation factor 2. CD4-PE40 binds specifically to gp120 on the surface of HIV infected cells and kills these cells by inhibiting protein synthesis. Thus, this molecule is a potential therapeutic for AIDS. In combination with the antiviral drug, AZT, CD4-PE40 can eliminate HIV from infected cultures (Ashorn et al., *Proc. Natl. Acad. Sci. USA* 87:8889 (1990)). Thus, CD4-PE40 is currently being evaluated as a therapeutic agent for the treatment of AIDS in clinical trials.

Recombinant toxins made in *E. coli* are much smaller in size than conventional immunotoxins. Thus, they more easily penetrate tumors and tissues as compared with immunotoxins formed from more than one polypeptide strand. The single chain and small size make these toxins more effective in cell delivery. However, chimeric toxins are rapidly cleared from the blood. The short half-life of recombinant chimeric toxins could be a consequence of their small size or their sensitivity to proteases. For example, an improved CD4-PE40 toxin that readily penetrates tissue and has an increased half-life would directly benefit AIDS patients.

The half-lives of several therapeutic modalities have been improved using a variety of techniques such as those described by Collen et al., *Blood* 71:216-219 (1988); Hotchkiss et al., *Thromb. Haemostas.* 60:255-261 (1988); Tanswell et al, *Fibrinolysis* 3:79-84 (1989); Browne et al., *J. Biol. Chem.* 263:1599-1602 (1988); Wawrzynczak et al., *Mol. Immunol.* 29:213-220 (1992); Abuchowski *et al.*, *Cancer Biochem. Biophys.*

7:175 (1984) and Abuchowski *et al.*, *J. Biol. Chem.* 252:3582 (1977). Antibodies have been chemically conjugated to toxins to generate immunotoxins which have increased half-lives in serum as compared with unconjugated toxins and this increased half-life is attributed to the native antibody. Studies indicate that immunoglobulin has an increased circulation time as compared with other molecules of similar size. Pollock et al. (*Eur. J. Immunol.* 20:2021, 1990) reported that at least 30% of IgG remains in the circulation after 24-48 hrs and also that the subsequent decay rates of the IgG subclass is much slower than IgM or IgA subclasses. IgG1, IgG2a and IgG3 all have similar half-lives while IgG2b has a shorter half-life.

More recently, Capon et al. (*Nature* 337:525 (1988)) constructed hybrid molecules, termed CD4-immunoadhesins 2γ1 and 4γ1, that consist of the first two or all four immunoglobulin-like domains of CD4 fused to the constant regions of the human IgG1 heavy chain. Polyacrylamide gel analysis indicated that both immunoadhesins were disulfide linked dimers with structural similarities to immunoglobulin. Dimers tend to be expensive to make and are larger molecules which deter them from readily penetrating tissue. While the immunoadhesins functioned in the circulation to bind HIV, as a dimer, they had limited penetration into tissue.

Increasing the half-life of therapeutically valuable immunotoxins would be a significant improvement over the current state of the art. Therefore it is an object of this invention to increase the half-life of immunotoxins in the circulation while maintaining the ability of the toxin to penetrate tissue. Moreover, it is a further objective of this invention to increase the potency of the immunotoxin. Improvements to CD4-PE40, for example, are critically important to the development of AIDS therapeutics.

SUMMARY OF THE INVENTION

This invention relates to single chain recombinant proteins, comprising the following domains: (a) a cytotoxic domain; (b) a ligand binding domain; and (c) a peptide linking domains (a) and (b) comprising an IgG constant region domain

having the property of increasing the half-life of the protein in mammalian serum. Preferably the IgG constant region domain is CH2 or a fragment thereof. The cytotoxic domain is preferably *Pseudomonas exotoxin* A (PE) wherein domain Ia has been deleted from the PE. An exemplary and particularly useful ligand binding domain is from CD4 receptor.

The invention also includes recombinant DNA molecules operably encoding the recombinant proteins and host cells expressing the proteins.

Further included are methods for treating HIV infection in a human, comprising administering a therapeutically effective dose of the recombinant proteins. Pharmaceutical compositions comprising a therapeutically effective dose of the recombinant proteins in a pharmaceutically acceptable carrier are also contemplated.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic of expression vector pJB 403H encoding CD4-PE40 under the control of the bacteriophage T7 promoter. The junction of CD4 and domain II of PE40 is given in the expanded sequence above the construct section. The DNA sequence of the linker and the corresponding amino acids are abbreviated using the single letter code.

Figure 1B relates plasmid nomenclature to the protein product produced by each of the constructs. Each construct contains CD4 and PE40 together with various segments from the Fc portion of IgG1. The numbers in parentheses indicate those amino acids derived from each indicated domain.

Figure 2 provides the SDS-polyacrylamide gel electrophoretic pattern of the purified protein chimeras stained with Coomassie Blue. Molecular weight size markers (in daltons) are provided in the left margin.

Figure 3 graphically illustrates the difference between blood concentrations of CD4-CH2-PE40, CD-CH1CH2-PE40, CD4-CH3-PE40, CD4-CH2CH3-PE40, and CD4-PE40 over time. About 40μg I^{125} labeled protein was injected intravenously and amounts assayed at time points indicated.

Figure 4 contains three SDS polyacrylamide gels each containing CD4-PE40 and CD4-CH2-PE40 digested with various amounts of trypsin at pH 7.4 (Figure 4A) and at pH 5.0 (Figure 4B). Figure 4C represents equal amounts of both CD4-PE40 and CD4-CH2-PE40 mixed and incubated with indicated concentrations of trypsin. Proteins were run on 12.5% polyacrylamide gels and stained with Coomassie blue.

DETAILED DESCRIPTION

A dire need for an effective therapeutic agent to slow or reverse infection in AIDS has resulted in the production of recombinant soluble forms of human CD4. CD4 is a T-lymphocyte surface molecule and is the cell receptor for Human Immunodeficiency Virus (HIV). CD4 specifically binds HIV envelope glycoprotein gp120. Few of the CD4 based candidates neutralize HIV infectivity to cell surface CD4. Initially we demonstrated the efficacy of a recombinant protein containing the HIV binding portion of human CD4 linked to a 40kD portion of the *Pseudomonas* exotoxin A. This molecule was termed CD4-PE40 and was produced in *E. coli*. This chimeric molecule selectively inhibits protein synthesis in cells expressing the HIV envelope glycoprotein and is selectively toxic to cells expressing the HIV envelope glycoprotein gp120 (Chaudhary et al., *Nature* 335:369 (1988) incorporated by reference herein). CD4-PE40 is now in clinical trials.

While the CD4-PE40 toxin successfully kills HIV infected cells, it has a reduced half-life as compared with other circulating molecules such as immunoglobulins or recombinant dimer molecules comprising CD4 and the immunoglobulin constant region. A toxin with an increased half-life in the circulation or improved HIV-infected cell cytotoxicity would directly benefit HIV patients worldwide.

One preferred embodiment of the invention discloses the incorporation of fragments of immunoglobulin into the single chain recombinant chimeric protein CD4-PE40 to confer an increased half-life *in vivo*. These fragments are broadly termed "domains". A domain may be a designated region of a

molecule such as the CH3 domain of an immunoglobulin, or domain can refer to fragments thereof. Since CD4-PE40 is typically given to patients intravenously, its circulatory half-life and rate of degradation plays an important role in its therapeutic efficacy. This invention dramatically improves the half-life of an important therapeutic.

The single chain recombinant proteins of the invention are produced by fusing through recombinant means, such as through the production of single chain antibodies in *E. coli*, a cytotoxic domain, a ligand binding domain and an IgG constant region domain peptide linking the first two domains. A variety of cytotoxic molecules are suitable for use as the cytotoxic domain in the recombinant immunotoxins described here. Any toxin known to be useful as the toxic component of an immunotoxin may be used so long as it is a protein that may be recombinantly expressed. Particularly useful as the cytotoxic domain are *Pseudomonas* exotoxin A; diphtheria toxin and ribosome inactivating toxins derived from plants and fungi, including ricin, sarcin, tricanthosin, saporin and others described in *Genetically Engineered Toxins*, ed. A. Frankel, Marcel Dekker, Inc. (1992), incorporated by reference herein; and any recombinant derivatives of those. See generally, "Chimeric toxins," Olsnes and Pihl, *Pharmac. Ther.* 25:355-381 (1982) and U.S. Patent Nos. 4,675,382 and 4,894,443 which describe fusion proteins containing diphtheria toxin fragments; all incorporated by reference herein. The cytotoxic domain from PE preferably comprises a PE molecule in which domain IA has been deleted, such as in PE-40. In addition, the PE cytotoxic domain can be further modified using site-directed mutagenesis or other techniques known in the art, to alter the molecule for particular desired application. Means to alter the PE molecule in a manner that does not substantially affect the functional advantages provided by the recombinant proteins described here can also be used and such resulting molecules are intended to be covered herein.

For maximum cytotoxic properties of a preferred PE domain, several modifications to the molecule may be used. An appropriate carboxyl terminal sequence to the recombinant molecule is preferred to translocate the molecule into the cytosol of target cells. Amino acid sequences which have been found to be effective include, REDLK (as in native PE), REDL or KDEL, repeats of those, or other sequences that function to maintain or recycle proteins into the endoplasmic reticulum, referred to here as "endoplasmic retention sequences". See, for example, Chaudhary *et al, Proc. Natl. Acad. Sci. USA* 87:308-312 and Seetharam *et al, J. Biol. Chem.* 266: 17376-17381 (1991) and commonly assigned, USSN 07/459,635 filed January 2, 1990, all of which are incorporated by reference herein.

A "ligand binding domain" refers generally to all molecules capable of reacting with or otherwise specifically recognizing or binding to a receptor on a target cell. Examples of such binding domains include, but are not limited to antibodies, growth factors such as TGFα, IL2, IL4, IL6, IGF1 or CD4, hormones and the like which specifically bind desired target cells.

Ligand binding domains from the CD4 receptor are preferred and are known and described in the art. See, e.g. Chaudhary et al., *Nature, supra.*; P.J. Maddon et al., *Proc. Natl. Acad. Sci.* USA, 86:9155-9157 (1987); Smith et al., *Science* 238: 1704-1707 (1987); Fisher et al., *Nature* 331:76-78 (1988); Hussey et al., *Nature* 331: 78-81 (1988); Deen et al., *Nature* 331:82-84 (1988); Trunecker et al., *Nature* 331:84-86 (1988); and Berger et al., *Proc. Natl. Acad. Sci. USA* 85:2357-2361 (1988); all incorporated by reference herein. Preferably, a binding domain including about 180 amino acids from the N-terminal end of CD4 is used.

The amino terminal end of an IgG molecule is characterized by sequence variability in both the heavy (V_H) and light (V_L) chain regions. The rest of the molecule is relatively constant. The constant portion of the light chain is termed the C_L region and the constant portion of the heavy chain is further divided into three structurally discrete

regions: CH1, CH2 and CH3. The hinge region is a segment of heavy chain between the CH1 and CH2 domains. See Roitt et al., *Immunology*, Harper & Row Publishers, New York City, New York (1989). IgG constant region domains are useful in the invention and are preferably of human origin. The regions may be chimeric (Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)) or humanized (Jones et al., *Nature* 321:522-525 (1986)).

The IgG constant region domains useful in the proteins here include all or any one of the constant regions, CH1, CH2 and CH3, any fragments thereof and any combination of these. Exemplary sequence listings for these regions are known and are found in Ellison et al., *Nuc. Acid Des.*, 10:4071-4079 (1982), incorporated by reference herein.

Preferably the IgG constant region domains incorporated into the recombinant proteins will be CH2, CH3, CH2-CH3, or CH1-CH2, most preferably CH2. Fragments of these domains may alternatively be used. Effective fragments will be those that have the property of increasing the half-life of the ligand binding domain in mammalian serum.

Introduction of an IgG constant region domain into the proteins disclosed here increases the half-life of the product in mammalian serum. An increase in the half-life may be demonstrated by using the method described in Example 3 below. Preferably the IgG constant region domain will increase the half-life of the protein in serum by about 200%, most preferably by about 1000%.

Recombinant or fusion proteins of the invention may be expressed in a variety of host cells, including *E. coli*, other bacterial hosts, yeast, and various higher eucaryotic cells such as the COS, CHO and HeLa cells lines and myeloma cell lines. The recombinant protein gene will be operably linked to appropriate expression control sequences for each host. For *E. coli* this includes a promoter such as the T7, trp, or lambda promoters, a ribosome binding site and preferably a transcription termination signal. For eucaryotic cells, the control sequences will include a promoter and preferably an enhancer derived from immunoglobulin genes,

SV40, cytomegalovirus, etc., and a polyadenylation sequence, and may include splice donor and acceptor sequences. The plasmids of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation for *E. coli* and calcium phosphate treatment or electroporation for mammalian cells. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the amp, gpt, neo and hyg genes.

Once expressed, the recombinant fusion proteins can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (*see*, generally, R. Scopes, *Protein Purification*, Springer-Verlag, N.Y. (1982)). Substantially pure compositions of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity are most preferred for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically.

The recombinant fusion proteins and pharmaceutical compositions of this invention are particularly useful for parenteral administration such as intraperitoneal administration or intravenous administration. The compositions for administration will commonly comprise a solution of the fusion protein dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, *e.g.*, buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of fusion protein in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like

in accordance with the particular mode of administration selected and the patient's needs.

Thus, a typical pharmaceutical composition for intravenous administration would be about 0.5 to 10 mg per patient per day. Dosages from 0.2 up to about 30 mg per patient per day may be used. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as *Remington's Pharmaceutical Science*, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980).

The compositions containing the present fusion proteins or a cocktail thereof (i.e., with other proteins) can be administered for therapeutic treatments. In therapeutic applications, compositions are administered to a patient suffering from a disease, such as AIDS or to those patients positive for HIV. An amount adequate to accomplish this is an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health.

Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the proteins of this invention to effectively treat the patient.

Additional General Definitions

"Recombinant" means that the subject product is the result of the manipulation of genes into new or non-native combinations.

A "vector" is a sequence of DNA, typically in plasmid or viral form, which is capable of replicating in a host. A vector can be used to transport or manipulate DNA sequences. An "expression vector" includes vectors which are

capable of expressing DNA sequences contained therein, producing a protein product. The coding sequences are linked to other sequences capable of effecting their expression, such as promoters and enhancers.

The term "without significant cytotoxicity" means that the fusion protein of the present-invention does not affect the function of the untargeted cells to any appreciable degree or to any abnormal level.

The following examples are offered by way of illustration and are not to be construed as limiting the invention as claimed in any way.

EXAMPLES

MODIFICATIONS TO INCREASE THE HALF-LIFE OF CD4-PE40

Vector Construction

The constructs used to generate chimeric toxins with altered half-lives illustrated in Figure 1, were prepared by modifying pJB 403H. Plasmid pJB 403H is a modification of pVC403 deposited with the American Type Culture-Collection, Rockville, Maryland which bears ATCC deposit No. 67739, (Chaudhary *et al.*, *Nature* 335:368 (1988), incorporated by reference). Plasmid pVC403 contains a fusion gene encoding the first 178 amino acids of mature CD4 and amino acids 1-3 and 253-613 of PE. The gene is under control of a T7 late promoter. Plasmid pJB 403H resulted from a modification of pVC403 such that there is a Hind III site at the junction of CD4 and PE40 which has been used for various insertions. The primers were designed using the sequence published by Ellison et al. (*Nucl. Acids Res.* 10:4071 (1982) incorporated by reference herein). Exemplary primers used in this study are provided below.

TABLE 1

JKB-1 5' AGCCTCCACCAAGGGCCCATCGGTCTTCCC 3' (SEQ. I.D. NO. 1)

JKB-2 5' TCATTTACCCGGAGACAGGGAGAGGCTCTTCTG 3' (SEQ. I.D. NO. 2)

JKB-3 5' TCCATCTCTTAAGCTTCACCTGAGCTCCTGGGGGGACCG 3' (SEQ. I.D. NO. 3)

JKB-4 5' CCCACGGGTCCAAGCTTTGGCTTTGGAGATGGTTTTCTCG 3' (SEQ. I.D. NO. 4)

JKG-35 5' AACCTCTGTAAAGCTTCGCAGCCCCGAGAACCACAG 3' (SEQ. I.D. NO. 5)

JKG-33 5' CGGCCGTCGCAAAGCTTTACCCGGAGACAGGGAGAG 3' (SEQ. I.D. NO. 6)

JKG-15 5' ACCACCTCTCTTAAAGCTTCCACCAAGGGC 3' (SEQ. I.D. NO. 7)

JKG-13 5' CCCTCCCTGTGCGAGCTCAGGTGCAACTTTCTTGTCCACCTT 3' (SEQ. I.D. NO. 8)

* denotes the location of HindIII sites and + a SacI site.

JKB-1 and JKB-2 (SEQ. I.D. NO. 2) were used to generate the full length IgG constant region fragments by polymerase chain reaction (PCR) using human IgG1 cDNA as template. From this constant region, using the remaining primers the Fc immunoglobulin fragments CH2, CH3, CH1-CH2 and CH2-CH3 were generated that were then positioned between CD4 and PE40 as illustrated in Figure 1B. Example 1 provides specific methods used to generate plasmids pJB 403C2, pJB 403C3, pJB 403C12 and pJB 403C23. These plasmids are modified versions of pJB 403H (Figure 1A) that incorporate the inserts provided in Figure 1B. The recombinant chimeric toxins produced from each of these plasmids are modified forms of CD4-PE40. The parent toxin CD4-PE40 together with its modified forms are generally referred to herein as "CD4-PE40 and its derivatives".

All of the chimeric proteins were expressed in *E. coli* strain BL21 (λDE3), which carries an inducible T7 RNA polymerase gene on a prophage. (Studier and Moffatt, *J. Mol. Biol.* 189:113-130 (1986)). Expressed protein was present in bacterial cell inclusion bodies. The inclusion bodies were dissolved in guanidine hydrochloride, and, after renaturation, the recombinant proteins were purified by successive ion exchange and size exclusion chromatography as described by Batra et al., *Mol. Cell. Biol.* 11:2211 (1991), which is hereby incorporated by reference. The generation of plasmids, the location of the expressed protein and the methods used to isolate and purify the recombinant protein are known in the art. Depending on the plasmid, the location of the expressed recombinant protein may vary. Thus these methods are exemplary and should not detract from the scope of this invention.

<u>Activity of the Modified Toxin in Culture</u>

The purity of the various chimeric toxins is shown in Figure 2. Protein was assayed by Bradford's method using Pierce

Coomassie blue Plus Reagent (Pierce Chemical, Rockford, Illinois and Bradford et al., *Anal. Biochem.* 72:248, (1976)). Samples were run on 10% SDS polyacrylamide gels (SDS-PAGE) and were judged to be about 90% pure. Purity is expected to vary between preparations, thus those preparations judged to be substantially pure were tested in culture.

Following purification, the activities of each of the purified toxins was assayed on ENV-15 cells from the Upjohn Company, Kalamazoo, MI, a transfected CHO cell line that expresses gp160 on its surface, or on CV1 cells, also expressing the HIV envelope glycoprotein. The HIV envelope glycoprotein was introduced into CV1 cells using a recombinant vaccinia virus as described in Example 2. On both cell types, all five chimeric toxins were active and had similar cytotoxic activities (Table 2). Further, the chimeric toxins had no activity on uninfected cells. The uninfected controls were not killed demonstrating the specificity of CD4-PE40 and its derivatives for HIV envelope glycoprotein 120.

TABLE 2

Cytotoxicity of CD4-PE40 and its Derivatives on Target Cells

	ID50 (ng/ml)		
Protein	CV1 cells		ENV15
	gp160+	gp160*	gp160+
CD4-PE40	0.5	>100	17
CD4-CH2-PE40	0.7	>100	19
CD4-CH3-PE40	0.8	>100	ND
CD4-CH1-CH2-PE40	0.5	>100	ND
CD4-CH2-CH3-PE40	1.2	>100	ND

*There was no inhibition of protein synthesis at the concentration indicated; these cells do not express gp120.

Half-Life Determination in Rats

The half-life of CD4-PE40 and its derivatives was determined by intravenously injecting labelled protein into rats. Purified CD4-PE40, CD4-CH2-PE40, CD4-CH3-PE40, CD4-CH1-CH3-PE40, and CD4-CH2-CH3-PE40 were expressed, purified and labelled with ^{125}I. The labelled protein was injected intravenously into rats at a concentration of 20-50 $\mu g/kg$.

Serial plasma samples were collected and assayed for TCA precipitable ^{125}I. Averaged counts were translated into protein concentrations and half-lives were determined using computer program RSTRIP (See Example 3) that employed a biexponential equation as described in Example 3. As shown in Table 3, the mean residence time in the circulation was highest for CD4-CH2-PE40 (115 min) whereas the lowest residence time was obtained from CD4-PE40 (47 min). The α and β half-lives of CD4-PE40 were 6.3 and 40 min respectively whereas those for CD4-CH2-PE40 were prolonged to 24 and 122 minutes (Table 3) The mean residence time of CD4-CH3-PE40, CD4-CH2-CH3-PE40 and CD4-CH2-CH3-PE40 was found to be 78, 89 and 98 minutes respectively (Table 3) which was longer than CD4-PE40 and shorter than that for CD4-CH2-PE40. The plasma clearance of CD4-CH2-PE40 and CD4-CH2-CH3-PE40 was 1.0 and 1.4 ml/min/kg, respectively, compared to CD4-PE40 at 1.9 ml/min/kg (Table 3). A comparison of toxin concentration to clearance time for CD4-PE40, CD4-CH2-PE40, CD4-CH1CH2-PE40, CD4-CH3-PE40, and CD4-CH2CH3-PE40 is provided in Figure 3. These results indicate that the insertion of CH2 into the CD4-PE40 construct increased the serum half-life of the CD4 containing toxin.

TABLE 3

Plasma pharmacokinetics of ^{125}I-labeled CD4-PE40 and various derivatives in rats.

Protein	Mean Residence Time (min)	Clearance (ml/min/kg)	t1/2α (min)	t 1 / 2 β (min)
CD4-PE40	47+2	1.90+0.10	6.0+2.0	40+5
CD4-CH2-PE40	115+20	1.00+0.01	24.5+5.4	122+36
CD4-CH3-PE40	78+4	1.31+0.02	12.9+0.3	80+2
CD4-CH1-CH2-PE40	98+23	1.16+0.14	14.3+5.2	99+32
CD4-CH2-CH3-PE40	89+9	1.43+0.14	10.6+0.6	79+5

<u>Sensitivity to Protease Digestion</u>

Digestion of PE with trypsin at neutral pH results in the generation of 55 kD, 37 kD, and 28 kD fragments (Ogata et al. *J. Biol. Chem.* 265:20678 (1990). However, at an acidic pH only the 37 kD and 28 kD fragments are observed (Jiang et al., *J. Biol. Chem.* 265:8636 (1990)). Incubation of CD4-PE40 *in vitro* with trypsin at neutral pH also resulted in the generation of a 37 kD and a 28 kD fragment. The N-terminal sequence of the 37 kD fragment was found to be Met-x-Glu-Pro-Leu-Gly-Glu-Glu-Glu- Tyr/Leu. The N-terminal sequence of this fragment suggests that the cleavage site is at Arg 274 or Arg 276 in domain II of PE.

One possible explanation for a difference in the plasma half-lives of CD4-PE40 and its derivatives could be their susceptibility to intravascular proteolysis. Thus the chimeric proteins containing the half-life enhancing modifications were tested for their sensitivity to protease degradation. As an example, a comparison was made of the sensitivities of CD4-PE40 and CD4-CH2-PE40 to trypsin degradation. Other proteases including thrombin and plasmin were similarly used to determine the propensity of the constructs to proteolytic degradation. (Table 4). Since PE40 is a potent toxin it is important to minimize those proteolytic events that could free the toxin domain from the targeting domain. Therefore the constructs should be screened against a number of enzymes. Figure 4 illustrates the susceptibility of CD4-PE40 and CD4-CH2-PE40 to increasing concentrations of trypsin. Samples of CD4-PE40 and CD4-CH2-PE40 were digested with trypsin at pH 7.4 and pH 5. At pH 7, CD4-CH2-PE40 was significantly more resistant to trypsin degradation than CD4-PE40 (Figure 4A). Similar size fragments were produced by trypsin digestion with both CD4-PE40 and CD4-CH2-PE40 indicating that the cleavage sites were the same in both molecules. It is known that the *Pseudomonas* toxin loses its secondary structure at pH 5. Since both CD4-PE40 and CD4-CH2-PE40 were proteolyzed by trypsin to the same degree at pH 5, the proteolytic site was localized to PE40. Both molecules were completely digested at pH 5 using the 40 ng trypsin concentration (Figure 4B).

To determine if the CH2 domain was acting as a protease inhibitor that could inhibit proteolysis of molecules without the CH2 domain, equal concentrations of CD4-PE40 and CD4-CH2-PE40 were mixed and digested with different concentrations of trypsin. The presence of CD4-CH2-PE40 with CD4-PE40 did not inhibit CD4-PE40 digestion, therefore the CH2 domain did not confer protection to the CD4-PE40 chimera. The results are provided in Figure 4C. In this experiment, CD4-PE40 was completely digested at 40 ng whereas CD4-CH2-PE40 showed no sign of degradation using up to 200 ng of trypsin (Figure 4C). The CH2 domain is not acting as a nonspecific protease inhibitor. Similar results were obtained when CD4-PE40 and CD4-CH2-PE40 were incubated separately with thrombin and plasmin at pH 7.4 as shown in Table 4. Ten μg of protein was incubated at 20°C with thrombin for one hour and with plasmin for three hours. CD4-CH2-PE40 was found to be more resistant to thrombin and plasmin than CD4-PE40.

TABLE 4

Cleavage of CD4-PE40 and CD4-CH2-PE40 by Thrombin and Plasmin Proteolysis

Protein	Thrombin (unit)				Plasmin (unit)			
	0	0.5	1.0	2.0	0	0.5	1.0	2.0
CD4-PE40	-	-	+	++	-	++	++	++
CD4-CH2-PE40	-	-	-	++	-	-	++	++

(-) indicates no proteolysis (++) indicates complete proteolysis.

Since the postulated cleavage site is within the cytotoxic domain of the CD4-PE40 toxins, it is likely that at neutral pH CH4-CH2-PE40 is folded such that the protease sensitive sites in the disulfide loop of the molecule are masked by the CH2 domain rendering the molecule resistant to digestion, while at the acidic pH, those sites are exposed and therefore the molecule is sensitive to trypsin. Thus any peptide domain that protects these sites from proteolysis at neutral pH would be useful, and is therefore contemplated within the scope of

this invention. Similarly, the cytotoxic domain could be subjected to site-directed mutagenesis, using techniques well known in the art, to replace the protease sensitive sites with other amino acids that would render the CD4-PE40 toxin and its derivatives resistant to proteolysis.

In addition to the sequence of the toxin, the overall size of the chimera could influence its circulatory half-life. As previously noted, larger protein has reduced tissue permeability. Thus the size of the chimera could be modified using the half-life enhancing region to balance renal clearance and tissue permeability. The half-life enhanced CD4-PE40 derivative, CD4-CH2-PE40, may be mutagenized and additional sequences may be added to increase size. The reasons for selecting a particular half-life enhancing domain may therefore include, but are not limited to, the size of the polypeptide, the presence of proteolytic protecting domains, the ability to increase toxin potency and the permeability enhancing characteristics of the particular domain.

Toxicity of CD4-PE40 and its Derivatives

Concentrations of CD4-PE40 and CD4-CH2-PE40 were injected intraperitoneally into mice to assess their toxicity. The LD_{50} was determined after 96 hours for each protein at each dose. The LD_{50} of a single dose of CD4-PE40 was 60 μg and for CD4-CH2-PE40 it was greater than 80 μg. When the difference in molecular weight of these two molecules is considered, the results indicate that the insertion of CH2 into CD4-PE40 does not make the molecule more toxic to mice. Results of these studies are provided in Table 5.

Table 5

Toxicity of CD4-PE40 and CD4-CH2-PE40 in Mice

Amount injected (μg)	Number of deaths after 72 hrs. CD4-PE40	CD4-CH2-PE40
10	0/2 (168)	0/2 (140)
20	0/2 (336)	0/2 (280)
40	0/2 (672)	0/2 (560)
60	2/2 (1008)	0/2 (840)
80	2/2 (1344)	2/2 (1120)

The values in parentheses are the amount of protein injected in pmoles.

ENHANCING THE POTENCY OF CD4-PE40 AND ITS DERIVATIVES

It is additionally contemplated within the scope of this invention that the chimeric toxin CD4-PE40 and its derivatives can be further modified to alter one or more of the functional domains of this molecule. The CD4 region, the half-life enhancing region, if one is included, and the cytotoxic domain can all be altered using site-directed mutagenesis or PEglylation or other methods known in the art to improve the function of this chimeric toxin. Thus the CD4 region can be subjected to site-directed mutagenesis to alter or improve the binding properties of this molecule to gp120. Similarly, site-directed mutagenesis or substitution can be used to generate an improved cytotoxic domain.

Particular embodiments of the invention will be discussed in detail below. Reference has been made to possible variations within the scope of the invention. Those with skill in the art will recognize that there are a variety of alternative techniques and procedures known in the art which would similarly permit one to successfully perform the intended invention.

EXAMPLE 1

Cloning of Constant Domains
from Human IgG1 into CD4-PE40

To generate the constructs illustrated in Figure 1, the constant region of IgG was obtained from the human IgG_1 cDNA. A human spleen cDNA library was purchased from Clontech, Palo Alto, CA and cDNA for constant region of IgG1 was isolated using the polymerase chain reaction (PCR). The primers used to amplify human IgG1 constant region cDNA were designed using the sequence published by Ellison et al., *Nucleic Acids Res.* 10:4071-4079 (1982) incorporated by reference herein, and are provided in Table 1. A 30 cycle PCR was performed with denaturation at 94°C for 1 min., annealing at 55°C for 90 sec. and polymerization at 72°C for 2 min. with 10 sec. extension per cycle using a Perkin Elmer/Cetus thermocycler (Perkin-Elmer

Cetus Instruments, Norwalk, CT). Primers JKB-1 (SEQ. I.D. NO. 1) and JKB-2 (SEQ. I.D. NO. 2) were first used to amplify IgG1 constant region DNA using the spleen cDNA library as template. This amplified DNA was checked for size on an agarose gel using techniques well known in the art. Restriction analysis was performed to confirm fragment size and to check for the presence of Hind III restriction enzyme cleavage sites within the amplified fragments. Other domains were amplified and isolated using the primers in the following combinations; JKB-3 (SEQ. I.D. NO. 3) and JKB-4 (SEQ. I.D. NO. 4) for the CH2 domain, JKG-15 (SEQ. I.D. NO. 7) and JKG-13 (SEQ. I.D. NO. 8) for the CH1 domain, and JKG-35 (SEQ. I.D. NO. 5) and JKG-33 (SEQ. I.D. NO. 6) for the CH3 domain.

The amplified fragments were digested with HindIII and ligated to Hind III digested pJB 403H (Figure 1). The resulting ligation product contained segments of the constant region of IgG1 located between CD4 and PE40. A schematic of the constructs is provided in Figure 1B. pJB 403H was modified to include either the CH2 domain, the CH3 domain, or both the CH2 and CH3 domains. Plasmids pJB 403C2, pJB 403C3 and pJB 403C23 were generated by these ligations and encoded CD4-CH2-PE40, CD4-CH3-PE40 and CD4-CH2-CH3-PE40 respectively. To make plasmid pJB 403C12, encoding CD4-CH1-CH2-PE40, pJB 403H was digested with HindIII and BamH1. The DNA was dephosphorylated and the 4.2 kb DNA fragment was gel purified. The PCR amplified CH1 domain was digested with HindIII and SstI and a 330 bp fragment was gel purified. Plasmid pJB 403C2 DNA was digested with SstI and BamH1 and a 330 bp fragment containing the CH2 domain and part of domain II of PE were then gel purified. A three fragment ligation was performed with the 4.2 kb vector and the CH1 and CH2 domains.

The constructs were transformed into calcium chloride permeabilized BL21(λDE3) cells using techniques known in the art. Protein was expressed and isolated in the BL21(λDE3) cells as described by Batra et al., *Mol. Cell Biol.* 11:2200-2205 (1991).

EXAMPLE 2

Cytotoxic Assay of CD4-PE40 related constructs

Cytotoxic activities of CD4-PE40 and its derivatives were assayed on CV1 cells, available from the American Type Culture Collection, Rockville, Maryland expressing the HIV envelope glycoprotein using the assay designed for assessing CD4-PE40 cytotoxicity as described by Chaudhary et al., *Nature* 335:369-372 (1988) which is hereby incorporated by reference. CV1 cells were plated in 24 well plates and 24 hr later the cells were infected with vPE16, a recombinant vaccinia virus encoding gp160 (Chakrabarthi *et al.*, *Nature* 320:535 (1986) or Lipson *et al.*, *Nature* 323:725 (1986), both of which are incorporated herein) at an MOI of 20. The cultures were incubated for nine hours and toxin preparations were added. Four hours later the cells were exposed to ^{3}H-leucine (Amersham Corp., Arlington Heights, IL) for one hour. Radioactivity was measured in TCA precipitated protein fractions as described in Chaudhary et al., *Nature* 339:394 (1989) and results were expressed as a percentage of control where no toxin was added. The results of the cytotoxicity assays are provided in Table 2.

EXAMPLE 3

Plasma Pharmacokinetics

To determine the half-life of the products generated by the constructs illustrated in Figure 1B, 20-50 μg ^{125}I labeled protein was injected intravenously into rats and the amount of radioactivity was assayed in the TCA precipitable fraction over time. The labeled toxin was prepared by incubating 100 mg of protein in PBS, 0.5% Tween 80 with 500 μCu $Na^{125}I$ (Amersham, Arlington Heights, IL) in the presence of 10 μg iodogen (Pierce Chemical, Rockford, IL), previously dried on the bottom of a plastic tube, for 10 min on ice. ^{125}I-labeled protein was removed from unreacted iodide by passage over Sephadex G-25M (PD-10 column, Pharmacia, Piscataway, NJ) (Fraker et al., *Biochem. Biophys Res. Commun.* 80:849. (1978) which is hereby incorporated by reference). The iodination method used is known to be mild and results in little protein damage. In a

preliminary study to assess the purity of the labelled protein, SDS-PAGE autoradiography revealed a single major labeled band corresponding to CD4-PE40.

Male Sprague-Dawley rats (Charles River, Portage, MI), weighing approximately 250 g, were fitted with a chronic sampling cannula via the jugular vein. The rats were administered ^{125}I-labeled protein at a dose of 20-50 μg/kg. Serial blood samples were collected via cannula and the plasma was prepared for TCA precipitation. Plasma samples (100 μl) were diluted with 400 μl PBS and precipitated with an equal volume of cold 25% trichloroacetic acid (TCA). Following centrifugation, the pellet was radioassayed in a gamma counter. Since ^{125}I is not physiologically incorporated into protein outside the thyroid, and since the TCA does not precipitate proteins less than 5000 MW, the TCA precipitable radioactivity represented intact drug.

Plasma CD4-PE40 concentration-vs.-time data was evaluated by an exponential curve fitting program, RSTRIP (Version 5, MicroMath Scientific Software, Salt Lake City, Utah), which provided an estimate of A, B, a, and b by fitting concentration data (C) at time (t) to equation (1) where A and B are the calculated maximum concentrations (coefficients) for the functions described by the rate constants a and b. The half-life estimates, T1/2(a) and T1/2(b), and plasma clearance (CL) were obtained from equations (2) and (3). The area under the plasma concentration-time curve (AUC), the area under the first moment curve (AUMC), and the mean residence time (MRT) were determined by RSTRIP using equations (4), (5), and (6). The dose (D) was determined by the weight of the administered formulation.

Equations Used to Determine Half-Life

$$C = Ae^{-at} + Be^{-bt} \quad (1)$$
$$T1/2(a) = \ln 2/a,\ T1/2(b) = \ln 2/b \quad (2)$$
$$CL = D/AUC \quad (3)$$
$$AUC = A/a+B/b \quad (4)$$
$$AUMC = A/a^2+B/b^2 \quad (5)$$
$$MRT = AUMC/AUC \quad (6)$$

EXAMPLE 4

Sensitivity of CD4-CH2-PE40 to proteolysis

CD4-PE40 and CD4-CH2-PE40 were tested for their resistance to increasing concentrations of trypsin. To check the proteolytic processing, 40 μg of CD4-PE40 or CD4-CH2-PE40 were incubated with 40, 200 or 500 ng of trypsin at 25° for 60 minutes in PBS at either pH 7.4 or pH 5. An aliquot of the sample was then subjected to 12.5% reducing SDS-PAGE analysis (Figure 4A and 4B). When the digestion was carried out at pH 7, CD4-PE40 was completely degraded at 40 ng trypsin, whereas CD4-CH2-PE40 was resistant to trypsin at up to 200 ng. For N-terminal amino acid analysis, following electrophoresis, proteins were transferred onto PVDF membrane (Millipore), appropriate bands were cut out and sequenced using techniques well known to those with skill in the art.

To determine if the CH2 domain was acting as a protease inhibitor, and could inhibit proteolysis of molecules without the CH2 domain, equal concentrations of CD4-PE40 and CD4-CH2-PE40 were mixed and digested with different concentrations of trypsin.

Plasmin and thrombin were also used to compare the sensitivity of CD4-PE40 and CD4-CH2-PE40 to proteolytic cleavage. 10 μg of recombinant toxin was incubated at room temperature at pH 7.4 with different amounts of protease in a volume of 100 μl. The protein was incubated with thrombin for 1 hr and with plasmin for 3 hrs. At the end of the incubation, proteins were analyzed on 12.5% SDS-polyacrylamide gels and stained with Coomassie blue.

EXAMPLE 5

Toxicity in Mice

Concentrations of CD4-PE40 and CD4-CH2-PE40 were given intraperitoneally to mice to assess their toxicity *in vivo*. CD4-PE40 or CD4-CH2-PE40 was injected i.p. into five groups of female Balb/c mice in doses ranging from 10-80 μg. Animals were observed for 72 hrs. for signs of toxicity and death. The LD50 in these experiments was the dose of toxin that killed 50% of

the animals over the time indicated. In these studies, animals were observed for 96 hours.

EXAMPLE 6

Treatment of HIV Positive Patients with CD4-CH2-PE40

Patients positive for HIV will be administered CD4-CH2-PE40 in buffered saline intravenously at a dose of 10 μg/kg of patient body weight per day for 10 consecutive days. Progress will be measured by detecting to see if the CD4 positive cell population increases and by determining if there is a decrease in the viral antigens in the blood.

This invention provides methods for increasing the half-life of single chain recombinant toxins in the circulation. In addition, the invention identifies a particularly useful half-life enhancing CD4-PE40 derivative CD4-CH2-PE40. Moreover, methods are disclosed for further improving the half-life of the toxins using random and site-directed mutagenesis techniques. None of the derivatives tested altered the cytotoxic activity of the toxin as compared with the parent CD4-PE40 toxin. This work is the first example to prove that the CH2 fragment can be used to increase the half-life of a therapeutic recombinant single chain toxin.

While particular embodiments of the invention have been described in detail it will be apparent to those skilled in the art that these embodiments are exemplary rather than limiting, and the true scope of the invention is that defined in the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: UNITED STATES OF AMERICA, as Represented by the Secretary of the Department of Health and Human Services

(ii) TITLE OF INVENTION: RECOMBINANT TOXIN WITH INCREASED HALF-LIFE

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Townsend and Townsend Khourie and Crew
(B) STREET: Steuart Street Tower, One Market Plaza
(C) CITY: San Francisco
(D) STATE: California
(E) COUNTRY: US
(F) ZIP: 94105-1492

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: TO BE ASSIGNED
(B) FILING DATE: 12-AUG-1993
(C) CLASSIFICATION: TO BE ASSIGNED

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Weber, Ellen L.
(B) REGISTRATION NUMBER: 32,762
(C) REFERENCE/DOCKET NUMBER: 15280-29

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 543-9600
(B) TELEFAX: (415) 543-5043

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

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(B) LOCATION: 1..30
(D) OTHER INFORMATION: /function= "PCR primer"
/product= "JKB-1"
/note= "This primer is use to generate full length
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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

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(B) LOCATION: 1..33
(D) OTHER INFORMATION: /function= "PCR primer"
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constant region fragments."

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

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(B) LOCATION: 1..39
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/note= "Used to amplify the CH2 domain."

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..40
(D) OTHER INFORMATION: /function= "PCR primer"
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/note= "Used to amplify the CH2 domain."

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

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(B) LOCATION: 1..36
(D) OTHER INFORMATION: /function= "PCR primer"
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/note= "Used to amplify the CH3 domain."

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(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

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(B) LOCATION: 1..30
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/note= "Used to amplify the CH1 domain."

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

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/note= "Used to amplify the CH1 domain."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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42

<u>WHAT IS CLAIMED IS</u>:

1. A single chain recombinant protein, comprising the following domains:

(a) a cytotoxic domain;

(b) a ligand binding domain; and

(c) a peptide linking domains (a) and (b) comprising an IgG constant region domain having the property of increasing the half-life of said protein in mammalian serum.

2. The recombinant protein of Claim 1, wherein the IgG constant region domain is CH2 or a fragment thereof.

3. The recombinant protein of Claim 1, wherein the IgG constant region domain is CH3 or a fragment thereof.

4. The recombinant protein of Claim 1, wherein the IgG constant region domain is CH2-CH3 or a fragment thereof.

5. The recombinant protein of Claim 1, wherein the IgG constant region domain is CH1-CH2 or a fragment thereof.

6. The recombinant protein of claim 1, wherein the cytotoxic domain comprises *Pseudomonas exotoxin* A (PE).

7. The recombinant protein of claim 6, wherein domain Ia has been deleted from the PE.

8. The recombinant protein of claim 1, wherein the ligand binding domain is from CD4 receptor.

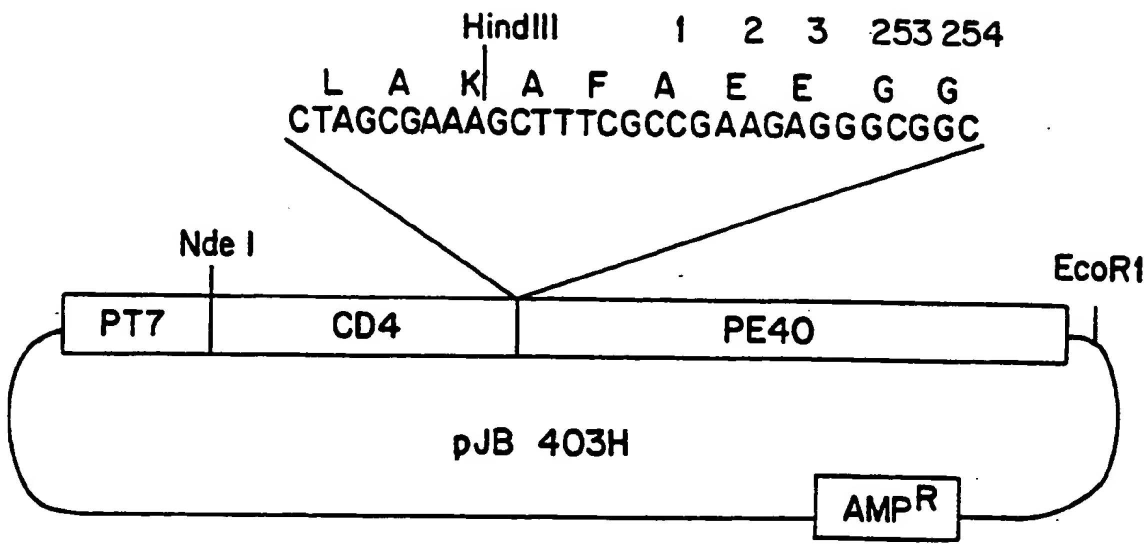
9. A recombinant DNA molecule operably encoding the protein of claim 1.

10. A host cell expressing the protein of claim 1.

11. A method for treating HIV infection in a human, comprising administering a therapeutically effective dose of the recombinant protein of Claim 1.

12. A pharmaceutical composition, comprising a therapeutically effective dose of the recombinant protein of Claim 1 in a pharmaceutically acceptable carrier.

13. The pharmaceutical composition of claim 12, wherein the recombinant protein comprises an IgG constant region domain of CH2 or a fragment thereof; cytotoxic domain from *Pseudomonas exotoxin* A; and the ligand binding domain from CD4 receptor.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Nde I
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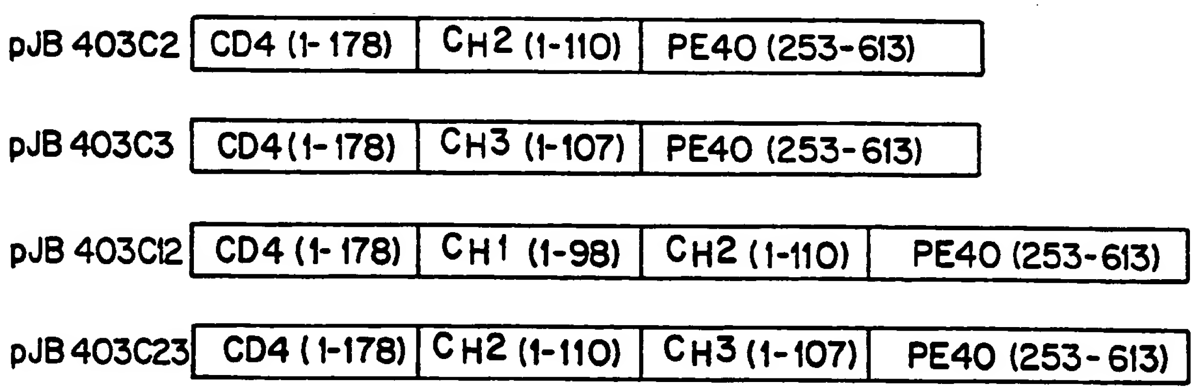
AMP R

FIG. 1A.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CH2 (1-110)
PE40 (253-613)
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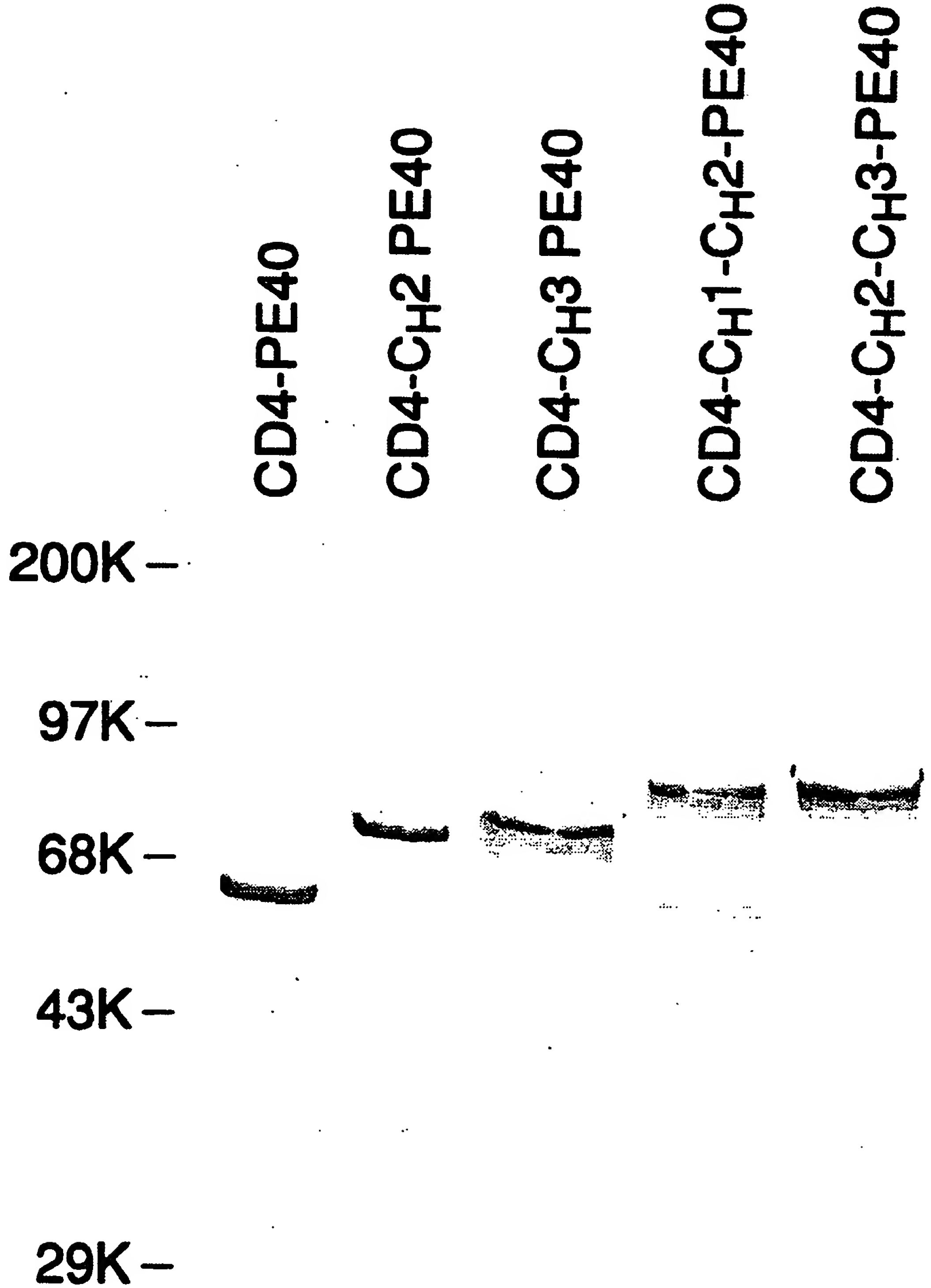
FIG. 1B.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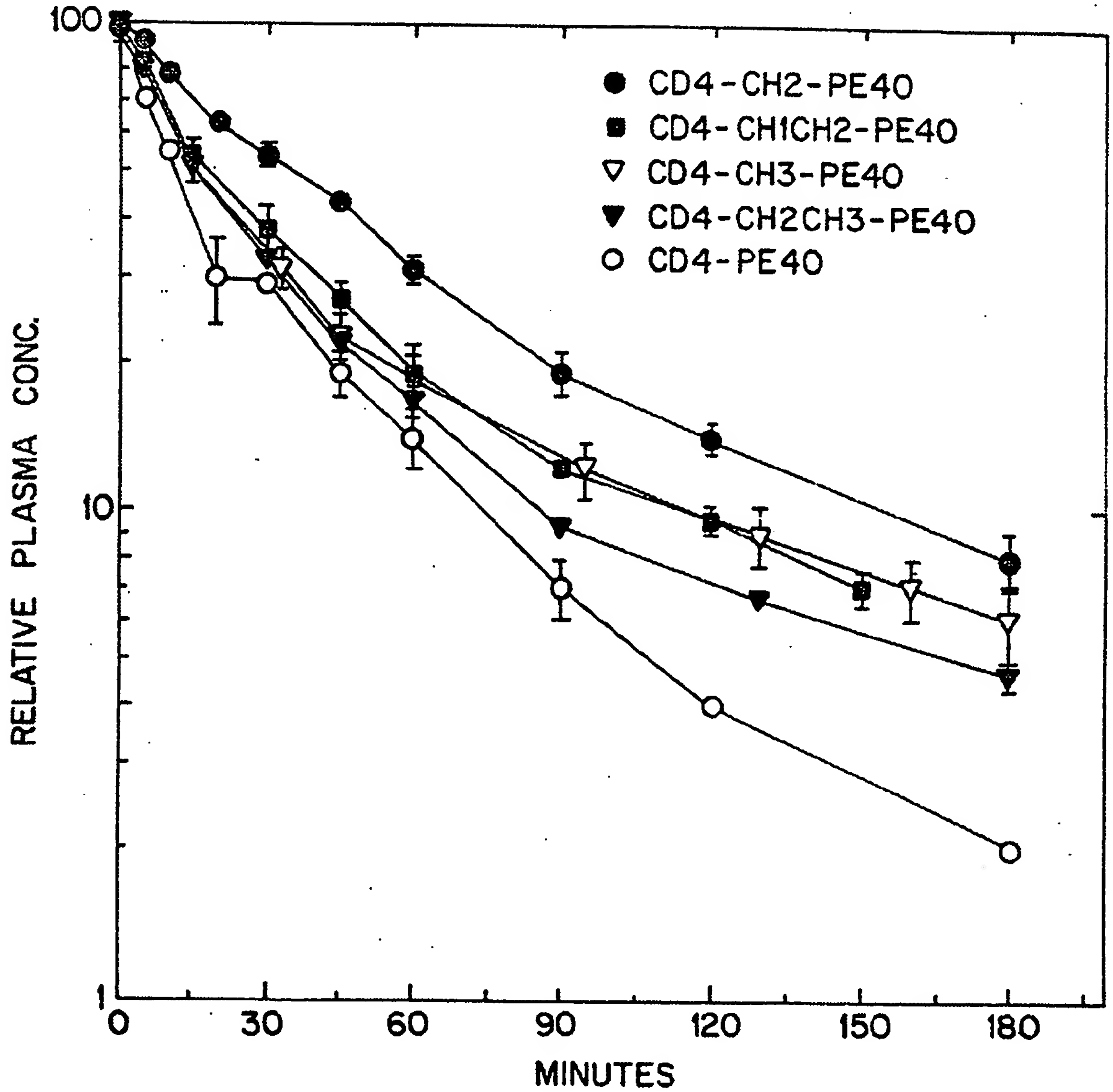
CD4-C_H2 PE40
CD4-C_H3 PE40
CD4-C_H1-C_H2-PE40
CD4-C_H2-C_H3-PE40
200K
97K
68K
43K
29K

FIG.2


100
10
1
RELATIVE PLASMA CONC.
0
30
60
90
120
150
180
MINUTES
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CD4-CH3-PE40
CD4-CH2CH3-PE40
CD4-PE40

FIG. 3.

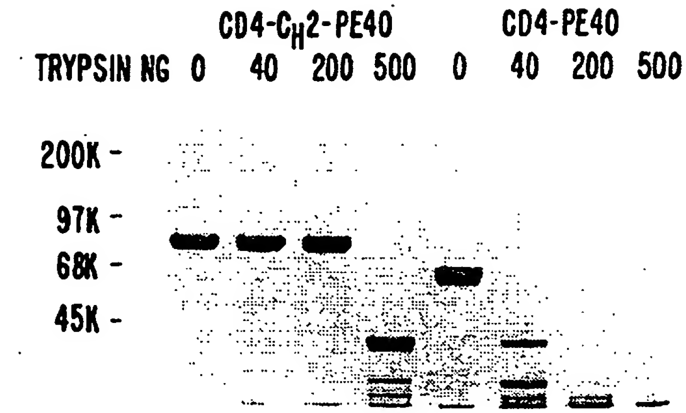

FIG.4A

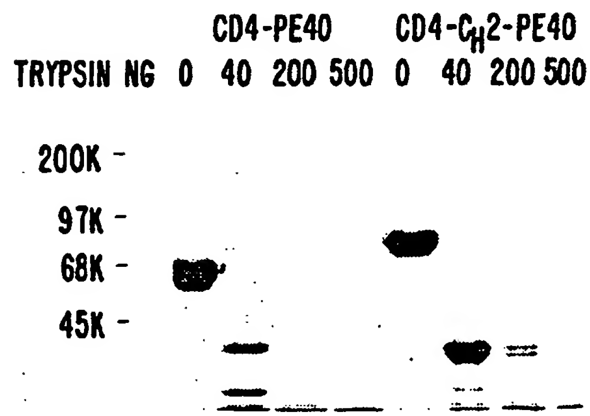

FIG.4B

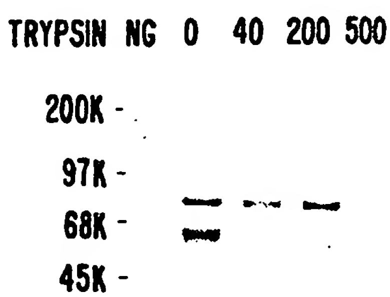

FIG.4C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 93/07672

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/62 C12N15/13 C12N15/31 C12N15/12 A61K37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,90 01035 (THE UNITED STATES OF AMERICA) 8 February 1990 see page 20 - page 27; figure 1 see page 36, line 3-6; figure 9	1-13
Y	EP,A,0 314 317 (GENENTECH, INC.) 3 May 1989 see example 6; table 4	1-13
A	WO,A,91 18099 (THE UNITED STATES OF AMERICA) 28 November 1991 see page 18, line 18 - line 31	1-13

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1993	18 -01- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

International application No.
PCT/US93/07672

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claim 11 is directed to a method of treatment of the human body, the search has been carried out and based on the alleged effects of the compound.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 93/07672

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9001035	08-02-90	US-A-	5206353	27-04-93
		AU-A-	4069089	19-02-90
		EP-A-	0428603	29-05-91
		JP-T-	3504802	24-10-91
EP-A-0314317	03-05-89	AU-A-	2557188	18-04-89
		EP-A-	0383799	29-08-90
		JP-T-	4502850	28-05-92
WO-A-9118099	28-11-91	AU-A-	7986891	10-12-91